1980

# The effects of transient myocardial ischemia on RNA synthesis in the reperfused rat heart.

Bruce Gregory. Young
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# THE EFFECTS OF TRANSIENT MYOCARDIAL ISCHEMIA ON RNA SYNTHESIS IN THE REPERFUSED RAT HEART

by

Bruce Gregory Young

A Thesis
submitted to the Faculty of Graduate Studies
through the Faculty of
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1980

To my wife Donna and
my Mother and Father

ABSTRACT

THE EFFECTS OF TRANSIENT MYOCARDIAL ISCHEMIA ON RNA SYNTHESIS IN THE REPERFUSED RAT HEART

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The effect of fifteen minutes of global ischemia and reperfusion was studied in the isolated perfused rat heart. Following fifteen minutes of ischemic arrest hearts were perfused with a Krebs-Henseleit bicarbonate buffer containing 15mM glucose and ($^3$H) uridine (0.1 uCi/ml). Total RNA was isolated with a phenol - SDS and a PCA extraction procedure for comparison. RNA isolated with the phenol - SDS extraction, demonstrated a rapid rate of ($^3$H) uridine incorporation during the initial 15 minutes of perfusion and then a slower linear rate of incorporation from 15 to 60 minutes, in the control hearts. An alteration in uridine incorporation was not detected in the ischemic reperfused hearts. When RNA was extracted from control hearts with PCA they exhibited a linear rate of ($^3$H) uridine incorporation from 5 to 60 minutes. Uridine incorporation in the ischemic reperfused hearts

demonstrated a 24% and 26% reduction after 30 and 60 minutes of perfusion respectively. Free intracellular uridine was increased throughout the perfusion period in the ischemic hearts.

To assess the effect of 15 minutes of global ischemia on RNA synthesis in isolated mycoytes the muscle cells were isolated from 30 minute perfused and reperfused hearts by enzymatic digestion. Myocytes from ischemic reperfused hearts exhibited a 62% reduction in uridine incorporation into RNA. The isolated cells had a 55% reduction of free intracellular uridine following reperfusion. These data demonstrates a discrepancy between the two RNA isolation procedures. The PCA extraction revealed a reduction in total tissue ($^{3}H$) uridine incorporation in the ischemic reperfused hearts. This decrease in RNA synthesis was even greater in the myocyte preparations and demonstrates that global ischemia has a deleterious effect on RNA synthesis during initial reperfusion periods.

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TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

## LIST OF APPENDICES

# CHAPTER I

## INTRODUCTION

The mammalian heart has a large reserve capacity to adapt to numerous environmental stresses (2, 4, 17, 37, 48). These stresses often result in growth of the myocardium. Experimentally, myocardial growth may be elicited with ventricular volume overload (44), catecholamine stimulation (46), anoxia (18) and chronic exercise (8, 37). The enhanced muscle growth is apparently associated with an increased size (hypertrophy) of the pre-existing muscle cell (myocyte). Claycomb (7) has indicated that the adult heart has lost its ability for DNA replication which suggests why the myocardium must adapt by hypertrophy.

The early biochemical response during pressure overload induced ventricular growth is an activation of nuclear transcription. Within a four hour period following aortic construction, an increase in RNA polymerase activity (45) and RNA synthesis (10) has been observed. Schreiber has shown an increase in contractile protein synthesis within a few hours of pressure overload (45). Therefore, it can be concluded that an increase in RNA and protein synthesis preceeds an increase

in heart weight during initial myocardial growth in which an increased RNA synthesis is one of the earliest biochemical changes (10, 16, 24, 30, 40, 41).

Of considerable importance is myocardial ischemia, a condition in which the heart receives a reduction in blood flow, resulting in a diminished oxygen and substrate supply (19). Chronic myocardial ischemia can result in an increase in myocardial mass which may lead to ventricular disfunction and subsequent heart failure (5, 48). The magnitude of the myocardial enlargement appears to be related to the severity and duration of the ischemic insult. At the present time, there is convincing pathological evidence linking coronary occulsion and ventricular growth (5, 48). The increase in heart mass secondary to myocardial ischemia is in part due to an increase in muscle cell hypertrophy.

Coronary occulsion may induce many structural as well as metabolic changes in the heart. Within a few seconds following occulsion of a coronary vessel, the affected area of the myocardium becomes cyanotic, as oxygen is rapidly extracted from hemoglobin and myoglobin (19, 20). The tissue shows decreased glycogen, and increased lactate concentration, mitochondrial swelling and a slight clumping of nuclear chromatin (8, 9). If the ischemic period is prolonged, cells degenerate and necrotic

damage appears (42). The ability of the cells to survive during reperfusion of the ischemic area is inversely related to the length of the ischemic insult (1, 23, 47). An ischemic interval of greater than 40 minutes generally results in an inability of the tissue to recover (8, 9, 23, 42). However, following an ischemic period of approximately 15 minutes, the heart quickly resumes normal functional and intracellular structural characteristics following reperfusion (42).

At the present time, the mechanisms of pathological myocardial growth are only partially understood. Studies using hypoxia (11) or anoxia (18) in attempts to simulate myocardial ischemia have shown that an increased RNA synthesis is one of the earliest biochemical changes during the recovery period. This seems to suggest that possibly ischemia may be a sufficient stimulus to activate the genetic material and cause subsequent tissue growth.

A problem encountered in many biochemical studies of the heart is the complexity and heterogenity of the organ (6). In the mature adult heart, myocytes are the primary functional cells but comprise only 20% to 25% of the total cell population (33). Neural, vascular and connective tissue make up the remainder of

the tissue mass. Presently, the development of isolation techniques for adult myocytes has permitted specific morphological and biochemical studies of the isolated myocyte (7, 12, 15, 34).

In the present study, the ability of the heart to synthesize ribonucleic acid following a single period of global ischemia were investigated. In addition, since the myocyte is functionally the most important cell in the heart, changes in myocyte RNA synthesis was also investigated.

# CHAPTER II

# METHODS

## Animal Care and Handling

Female Wistar rats, (200 - 220 grams), were housed in pairs and provided Purina Rat Chow and water ad libitum. The animals were randomly assigned to control and experimental groups.

## Removal of the Heart

Prior to perfusion, each animal was anesthesized with Nembutal (15 - 20 mg/animal), administered intraperitoneally. A midline incision was made and 200 units of heparin injected into the inferior vena cava. After 30 seconds, the heart was excised and placed in ice cold saline to arrest the contractions.

## Heart Perfusion

The heart was removed from the saline with fine tipped forceps and the aorta was slipped onto a grooved stainless steel cannula of a modified Langendorff perfusion apparatus and secured with a thread ligature. A

10 minute washout period was used to facilitate the removal of blood from the coronary vessels and to allow the heart to stabilize. The perfusion medium was a Krebs-Henseleit bicarbonate buffer (22) containing 15mM glucose. The perfusate was maintained at 37° C and gassed with 95% $O_2$ and 5% $CO_2$ continuously.

Induction of Myocardial Ischemia

To induce myocardial ischemia, the aortic cannula was clamped with a hemostat for 15 minutes and the heart allowed to come to an ischemic arrest. At the end of the ischemic period, the aortic clamp was removed and the heart transferred to a recirculating system and perfused at 60 mmHg with 30ml of perfusate containing 0.1uCi/ml ($^3H$) uridine (specific activity, 46 Cu/mmol).

Following the appropriate perfusion period, the heart was rapidly cut from the cannula, wrapped in tin foil and frozen in liquid nitrogen. The tissue was stored at -30° C.

Isolation of Total RNA

Method I (10)

Hearts were thawed to 0-4° C, trimmed of atria, greater vessels and connective tissue and finely minced

with scissors. The tissue mince was homogenized in 15 ml of 20mM Tris-HCl (pH 7.2) with a Potter Elvehjem tissue homogenizer. The homogenate was made 0.5% (W/V) with respect to sodium dodecylsulfate (SDS), shaken and left standing at room temperature for 5 minutes. An equal volume of 88% (V/V) phenol containing 0.1% (W/V) quinoline was added and the mixture was shaken on ice for 30 minutes. The sample was centrifuged at 10,000 x g for 10 minutes at 4° C. The upper aqueous phase was transferred to a cold test tube and re-extracted with 10ml of 88% phenol containing 0.1% quinoline. After centrifugation at 10,000 x g, the aqueous layer was removed and combined with one-tenth volume of 20% sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol. After mixing, the solution was allowed to stand for a minimum of 2 hours at -20° C. The precipitate was separated by 15 minutes of centrifugation at 10,000 x g.

The pellet was solubilized in 3ml of a medium containing 0.01 M Tris-HCl (pH 7.2), 0.02M $MgSO_4$ and 10ug/ml of DN'aseI (Sigma). The mixture was left at room temperature for 15 minutes. Ice cold distilled water (7ml) was added and the mixture made 0.5% (W/V) with respect to SDS. Five millilitres of 88% (V/V) phenol containing 0.1% quinoline was added and the mix-

ture was mechanically shaken on ice for 15 minutes. The suspension was centrifuged at 10,000 x g for 10 minutes and the upper aqueous phase containing the nucleic acids was precipated by the addition of one-tenth volume of 20% sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol. The mixture was allowed to stand overnight at -20° C. The nucleic acids were than pelleted at 10,000 x g for 15 minutes.

To remove the oligodeoxiribonucleic acids from the RNA, the following procedure was used. The RNA pellet was dissolved in 2ml of 20% sodium acetate (pH 5.2) and 2ml of 4M potassium acetate. One millilitre of ice cold 95% ethanol was added to the sample slowly and the mixture kept at -20° C for 30 minutes. After collecting the precipate at 10,000 x g for 10 minutes, the above procedure was repeated. The remaining pellet was transferred to conical tubes, washed three times with 95% ethanol and dissolved in 1ml of 2% sodium acetate (pH 5.2).

Method II (31)

Hearts were thawed to 0-4° C, trimmed of atria, greater vessels, connective tissue and weighed. The tissue was finely minced in 4ml of ice cold distilled water and homogenized with a Potter Elvehjem tissue

homogenizer. One millilitre of the homgenate was transferred to a conical centrifuge tube which contained 4ml of 0.5N perchloric acid (PCA) and the mixture allowed to stand on ice for 15 minutes. The sample was centrifuged at 600 x g. The resulting pellet was washed once with 0.5N PCA, twice with absolute ethanol and once with ether. The precipitate was extracted twice with 3ml of 0.5N PCA for 30 minutes at a temperature of 80° C. The final two extractions were combined and used for RNA, DNA and radioactive determinations.

Isolation of Myocytes

The isolation of myocytes followed the procedure of Glick et al. (12). Hearts were thawed to 0-4° C, trimmed of atria, great vessels and connective tissue and cut into small pieces of approximately $2mm^2$. The pieces were washed with a phosphate buffer containing 120mM NaCl, 10mM KCl, 5mM glucose, 0.4mM $NaH_2PO_4:H_2O$. The pH was adjusted to 7.4 using 200mM $Na_2HPO_4$. The tissue chunks were then placed into a 25ml Erlenmyer flask containing 3.5ml of phosphate buffer fortified with 1mg/ml collangenase (Sigma) and 2mg/ml hyaluronidase (Sigma). The flask was placed in a temperature controlled water bath (37° C) and

shaken for 30 minutes at a rate of 100 revolutions per minute. At the end of the first digestion period, the flask was removed and 10ml of room temperature phosphate buffer was added to facilitate myocyte collection. The samples were filtered through nylon mesh into an ice cold 50ml test tube. The remaining chunks were returned to the flask and the digestion repeated with 3.5ml of fresh enzyme-phosphate buffer. This process was repeated a total of five times. Since phase contrast examination indicated the first digestion contained primarily connective tissue and numerous damaged myocytes it was discarded and the following four digestions were combined.

Following each digestion, the cells were gently centrifuged at 37 x g for 5 minutes and the supernatant partially aspirated, taking care not to disturb the myocyte pellet. The cells were gently stirred into suspension and the next digestion was added to the tube. The final four dissociations were washed three times with enzyme free phosphate buffer and layered onto 10ml of 3% sucrose in phosphate buffer and centrifuged at 60 x g for 5 minutes. The myocytes were then washed three times with phosphate buffer. Each preparation was checked by phase contrast microscopy for contamination and structural integrity of the myocytes. Good

preparations were stored at -30° C in 3ml of phosphate buffer for subsequent analysis.

Isolation of Myocyte RNA

Isolated myocytes appear to resist standard procedures of tissue homogenization to liberate the nucleic acids. Numerous attempts to homogenize myocytes with Potter Elvehjem tissue homogenizer failed to release nucleic acids even when 0.5N PCA was used as a homogenizing medium. The following method proved to be most successful. Two millilitres of ice cold distilled water was added to the myocyte pellet. The suspension was homogenized with a polytron at a setting of 4, for two 30 second bursts. Eight millilitres of 0.5N PCA was added and the mixture allowed to precipitate overnight at 3° C. RNA was extracted following the procedure of Maggio (31) as stated above.

Determination of Free ($^3$H) Uridine in the PCA Soluble Fraction

In an attempt to determine the free radioactive uridine in the cytoplasm of the total heart and myocytes, the tissue was prepared and homogenized as stated previously. After the addition of 4 volumes of 0.5N PCA, the samples were allowed to precipitate on ice. Follow-

ing centrifugation at 600 x g for 5 minutes, 300ul aliquots was removed and used for radioactive determination.

RNA and DNA Determination

Total RNA was determined by the orcinol procedure of Schneider (44) using yeast RNA as standard. DNA content was determined by the diphenylamine procedure (5) using calf thymus DNA as standard. RNA values were corrected for DNA contamination by the method of Schneider (44).

Radioactive Determinations

Approapriate aliquots from the phenol isolation (100ul), PCA isolation (300ul) and PCA soluble fraction (300ul) were dissolved in 10ml of scintillation cocktail containing 4gm PPO and 50 mg POPOP per litre of toluene. Duplicate samples were counted for 10 minutes in a Beckman LS - 100 and the counts were corrected for background contamination. Counts for RNA samples were expressed as counts per minute per milligram of ribonucleic acid. The counts from the PCA soluble fraction were expressed as counts per minute per milligram of deoxyribonucleic acids.

Coronary Flows, Percent Water and Contraction Rate

To determine the rate of coronary flow, the

effluent perfusate was collected in a 10ml graduated cylinder during heart perfusion. The flow rates were expressed as millilitres per minute. Heart rates were determined by counting contraction rates visually for 10 seconds and expressing the rates as beats per minute. To calculate tissue percent water a 100mg piece of myocardium was removed from the apex following the appropriate perfusion period. The section was weighed, dried at 50° C for 72 hours and reweighed. The water content was expressed as the percent difference between the dry and wet weight.

Statistical Analysis

Due to the heterogeneous nature of the data, an unpaired "t"-test was used on the majority of the data. Welch's Correction for heterogeneous data was used to calculate degrees of freedom following the application of the "t"-test.

A one way analysis of variance was used to analyze the data from the comparison of Method I and Method II (Figure 2). Results of statistical analysis are located in Appendix G.

# CHAPTER III

# RESULTS

## Coronary Flow, Heart Rate and Tissue Percent Water

To determine if the hearts were able to maintain a stable hemodynamic function during recovery from ischemia, preliminary experiments on coronary flow, heart rate and tissue water content were completed. The data in Table 1 indicate that after 15 minutes of global ischemia, coronary flow was not significantly altered during the subsequent 30 minute perfusion. Similarily, the contraction rates and accumulation of tissue water were comparable in ischemic and control hearts. These results suggest that hemodynamic function was maintained in the ischemic hearts and that tissue edema was similar in both control and ischemic hearts.

## Effect of Perfusion Time on ($^3$H) Uridine Incorporation Into RNA Isolated with the Phenol Extraction Method

To determine the pattern of incorporation of uridine into RNA, hearts were perfused for 5, 15, 30 and 60 minutes with ($^3$H) uridine, 0.1 uCi/ml buffer (Figure 1). Total RNA was isolated, purified by the phenol - SDS method, and the radioactivity determined. The uridine incorporation increased rapidly during the

CORONARY FLOW RATES, CONTRACTION RATES AND TISSUE PERCENT WATER

TABLE 1

| | Percent Water Content | Contraction Rates (beats/min.) | Coronary Flow Rate (ml/Min.) |
|---|---|---|---|
| Ischemic | 80.50 ± .49 | 223 ± 5 | 14.31 ± 1.85 |
| Control | 80.90 ± .50 | 220 ± 5 | 15.54 ± 1.23 |

All points consist of data from 5 to 6 animals. Control hearts were perfused for 30 minutes, while the ischemic hearts were made ischemic for 15 minutes and reperfused for 30 mintues. The data represented in Table 1 was calculated at 30 minutes perfusion and the differences are not statistically significant (P .05). Each value is mean ± S.E.M.

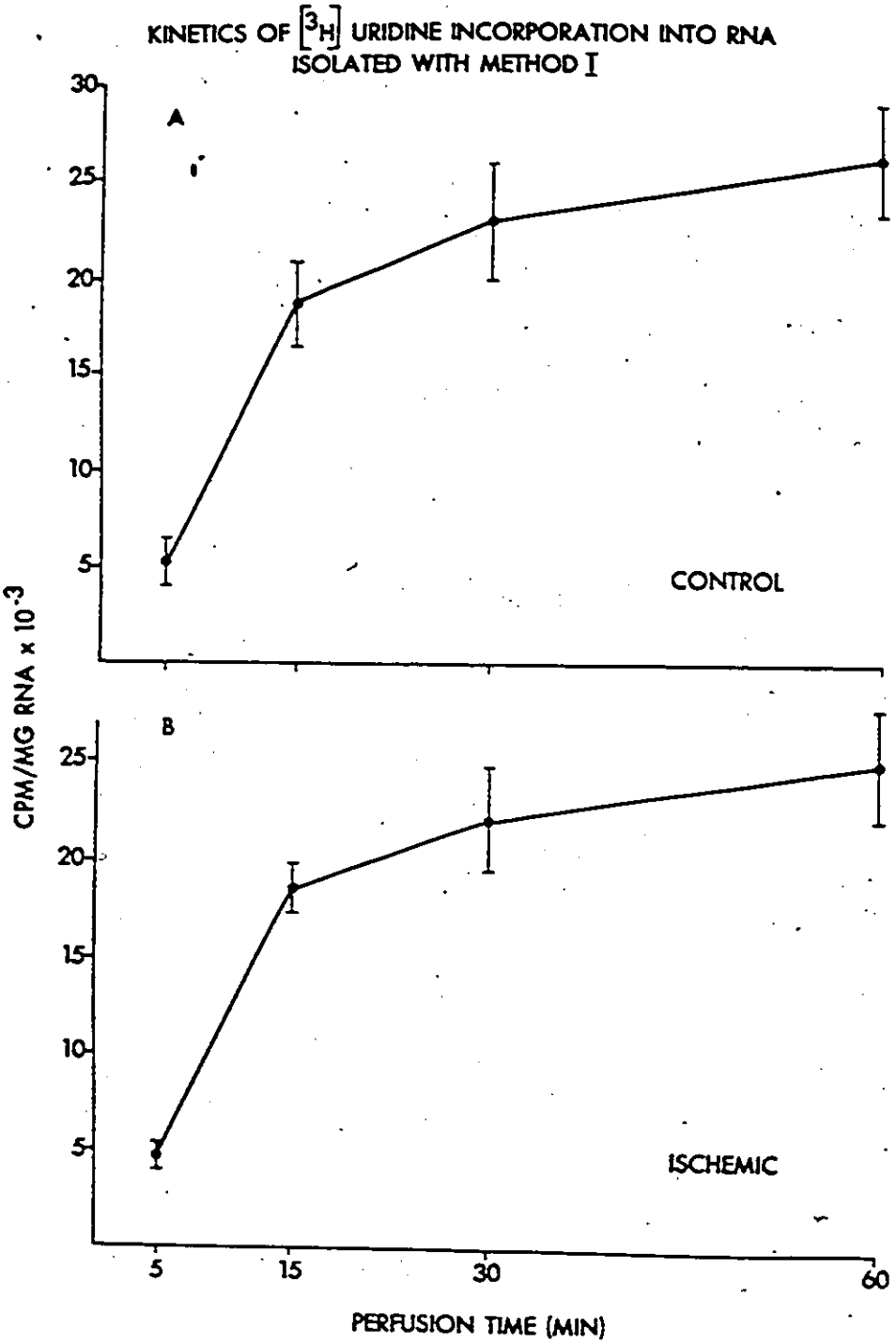


Figure 1. Effect of perfusion time on ($H^3$) uridine incorporation. Each value is mean ± S.E. of 4 - 8 animals.

initial 15 minutes of perfusion and then more slowly during 15 - 30 and 30 - 60 minutes of perfusion. Since there was not a significant difference ($P>.05$) between 30 and 60 minutes of perfusion all hearts were perfused for 30 minutes for the subsequent experiments.

Effects of Myocardial Ischemia and Reperfusion on RNA Synthesis

To determine the effect of global myocardial ischemia and reperfusion on RNA synthesis, the aortic cannula was cross clamped after 10 minutes prewash with non radioactive buffer (Figure 1b). Following 15 minutes of total occulsion, hearts were perfused for 5, 15, 30 and 60 minutes with ($^3$H) uridine 0.1 uCi/ml buffer. The RNA was extracted using the phenol - SDS extraction method and the radioactivity determined. As demonstrated in Figure 1b, the rate of RNA synthesis during the first 30 minutes of recovery was similar to the control hearts. After 60 minutes of reperfusion, RNA synthesis appeared slightly reduced in the ischemic hearts but this was statistically insignificant ($P>.05$).

Ribonucleic Acid Yields

Since there appeared to be a possible important biological reduction in RNA synthesis which was not statistically significant, one possibility could be

a marked reduction in the yield of RNA. Inspection of the time course data (Table 2) strongly suggests significant unexplainable low yields of RNA when using the phenol isolation. In an attempt to solve the problem, RNA was extracted using the PCA method and compared to the phenol - SDS procedure during 5, 15, 30 and 60 minutes of perfusion. The data in Table 2 clearly shows that the amount of RNA extracted with the PCA method was approximately 3 fold greater than the phenol - SDS procedure (Table 3). In addition, the yields were comparable to those reported by previous investigators using similar methodology (36). The yields following the phenol - SDS extraction were 75% of those reported by Posner (42) but were considerably lower than other previously reported yields using similar isolation procedures (10, 25).

The phenol extraction method provided inconsistant yields which may have lead to the large variability during the initial time course experiments. Furthermore, the variability in uridine incorporation could have resulted from a disproportionate sampling of labelled RNA as similarly noted by Lizardi (29) after following a similar procedure. From these data,

RNA YIELDS ISOLATED BY METHOD II

TABLE 2

| Treatment | PERFUSION TIME (Min.) 5 | 15 | 30 | 60 |
|---|---|---|---|---|
| Ischemic | 2,258.9 ± 140.1 | 2,383.9 ± 116.2 | 2,200.7 ± 61.2 | 2,202.1 ± 30.4 |
| Control | 2,360.5 ± 43.7 | 2,261.3 ± 48.1 | 2,330.3 ± 82.7 | 2,179.9 ± 137.3 |

Effect of perfusion time on RNA isolated by Method II. Mean values represent micrograms of RNA per gram of tissue wet weight ± S.E. N = 4.

RNA YIELDS ISOLATED BY METHOD I

TABLE 3

| Treatment | PERFUSION TIME (Min.) 5 | 15 | 30 | 60 |
|---|---|---|---|---|
| Ischemic | 970.4 ± 19.8 | 1171.3 ± 49.6 | 885.2 ± 29.6 | 952.9 ± 56.7 |
| Control | 726.4 ± 67.4 | 752.2 ± 27.5 | 872.4 ± 27.7 | 808.5 ± 53.7 |

Effect of perfusion time on yields of RNA isolated by Method 1. Mean values represent micrograms of RNA per gram of tissue wet weight ± S.E. N = 4 to 8.

it was decided to repeat the initial series of experiments using the alternative procedure for RNA extraction.

Comparison of Uridine Incorporation into RNA Isolated by the Phenol - SDS and PCA Extractions

To compare the amount of uridine incorporated into RNA using the two extraction procedures, twelve hearts were perfused for 30 minutes with ($^{3}H$) uridine, 0.1 uCi/ml buffer. The hearts were pooled, homogenized and treated as one sample. Eight aliquots were taken and isolated with the phenol extraction and eight aliquots were removed and the RNA isolated with the PCA extraction procedure. Figure 2 demonstrates the labelling of RNA when comparing the two methods and clearly demonstrates no significant difference between the two ($P>.05$). Aliquots isolated following the PCA method extracted 556.4ug/ml of homogenate while the phenol procedure extracted only 184.9ug/ml. Both methods provided workable amounts of RNA but the perchloric acid procedure isolated a higher proportion of the total RNA ($P<.005$). This factor combined with the previous observations led to the decision to repeat initial experiments using the PCA extraction method since the higher relative yields would reduce the possibility of losing some lower molecular weight RNA species.

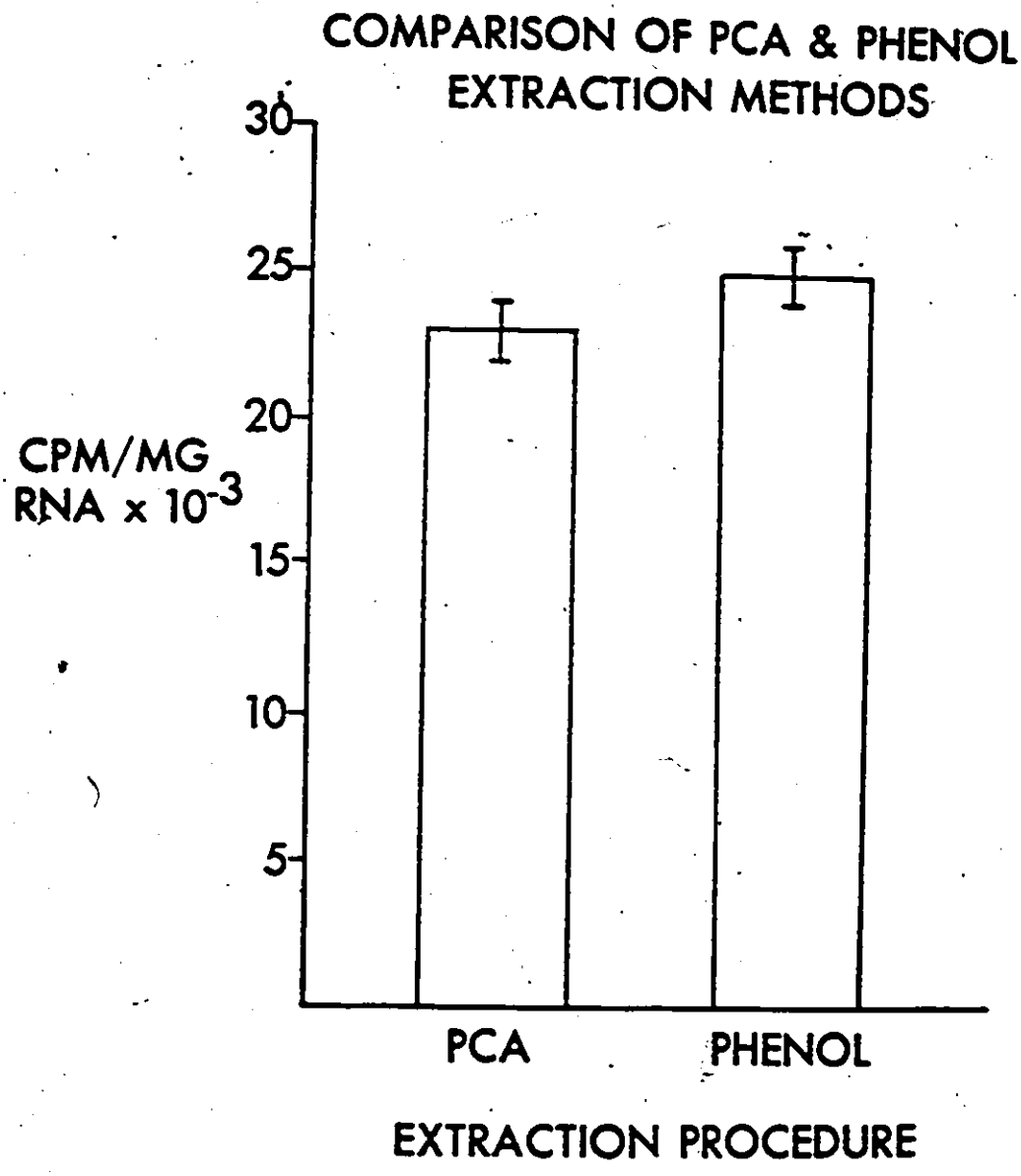


Figure 2. Twelve hearts were perfused for 30 minutes and pooled. Following homogenization eight aliquots were removed for each isolation procedure. Each value is mean ± S.E. of 7 - 8 animals.

## Effect of Perfusion Time and Myocardial Ischemia on ($^3$H) Uridine Incorporation into RNA Isolated With the PCA Extraction Method

### A) Control Hearts

To determine the pattern of uridine incorporation into RNA using the PCA method, hearts were perfused for 5, 15, 30 and 60 minutes with 0.1 uCi ($^3$H) uridine per ml of buffer and the RNA was isolated and radioactivity determined. Figure 3 demonstrates that uridine was incorporated linearly during the 60 minute perfusion period. This pattern differs considerably from that found with the phenol method (Figure 1a) as the 15 to 60 minute plateau phase was not detected.

### B) Ischemic Hearts

Following 15 minutes of global ischemia, the hearts were perfused for 5, 15, 30 and 60 minutes and the pattern of ($^3$H) uridine incorporation is shown in Figure 3. Ischemic hearts demonstrated a 24% ($P<.05$) and 26% ($P<.01$) reduction in uridine incorporation at 30 and 60 minutes respectively.

## ($^3$H) Uridine in the PCA Soluble Fraction in Total Myocardial Tissue

To determine the availability of free ($^3$H) uridine in the myoplasm present for RNA synthesis, control and ischemic hearts were perfused for 5, 15,

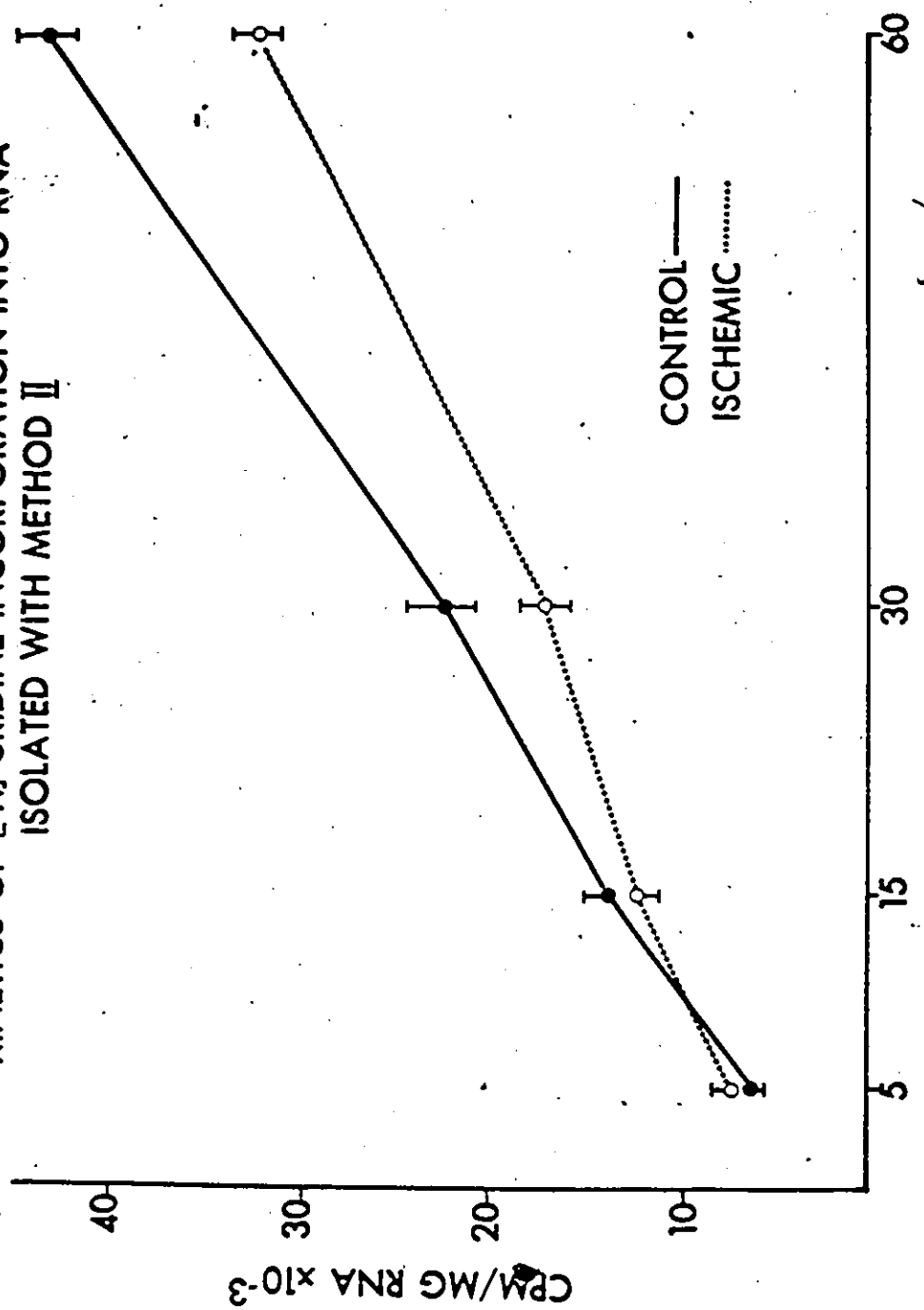


Figure 3. Effect of perfusion time on ($H^3$) uridine incorporation. Each value is mean ± S.E. of 4 animals.

30 and 60 minutes with ($^3$H) uridine 0.1 uCi/ml buffer. The radioactivity of the PCA soluble fraction was expressed as cpm/mg DNA as the DNA concentrations remained consistent throughout. The results are shown in Figure 4. Ischemic hearts exhibited an increase of 19%, 26%, 38% and 33% at 5, 15, 30 and 60 minutes of perfusion relative to control. Since the initial extracellular uridine concentration was initially maintained at 0.1 uCi/ml buffer it appears that the intracellular concentration of uridine increased in the ischemic reperfused hearts.

Comparison of ($^3$H) Uridine Incorporation into Isolated Myocytes

To determine uridine incorporation in a homgeneous population of ischemic and control myocytes, control and ischemic hearts were perfused for 30 minutes with standard buffer containing ($^3$H) uridine, 0.1 uCi/ml. Two hearts were pooled for each sample and myocytes were isolated according to the method of Glick at al. (12). The cells were examined under phase contrast to determine integrity at the completion of each isolation. Each preparation contained more than 80% intact cardiac cells as determined by random field examination.

Myocyte RNA was isolated with the PCA extraction method. Ischemic myocytes exhibited a 62% ($P<.001$)

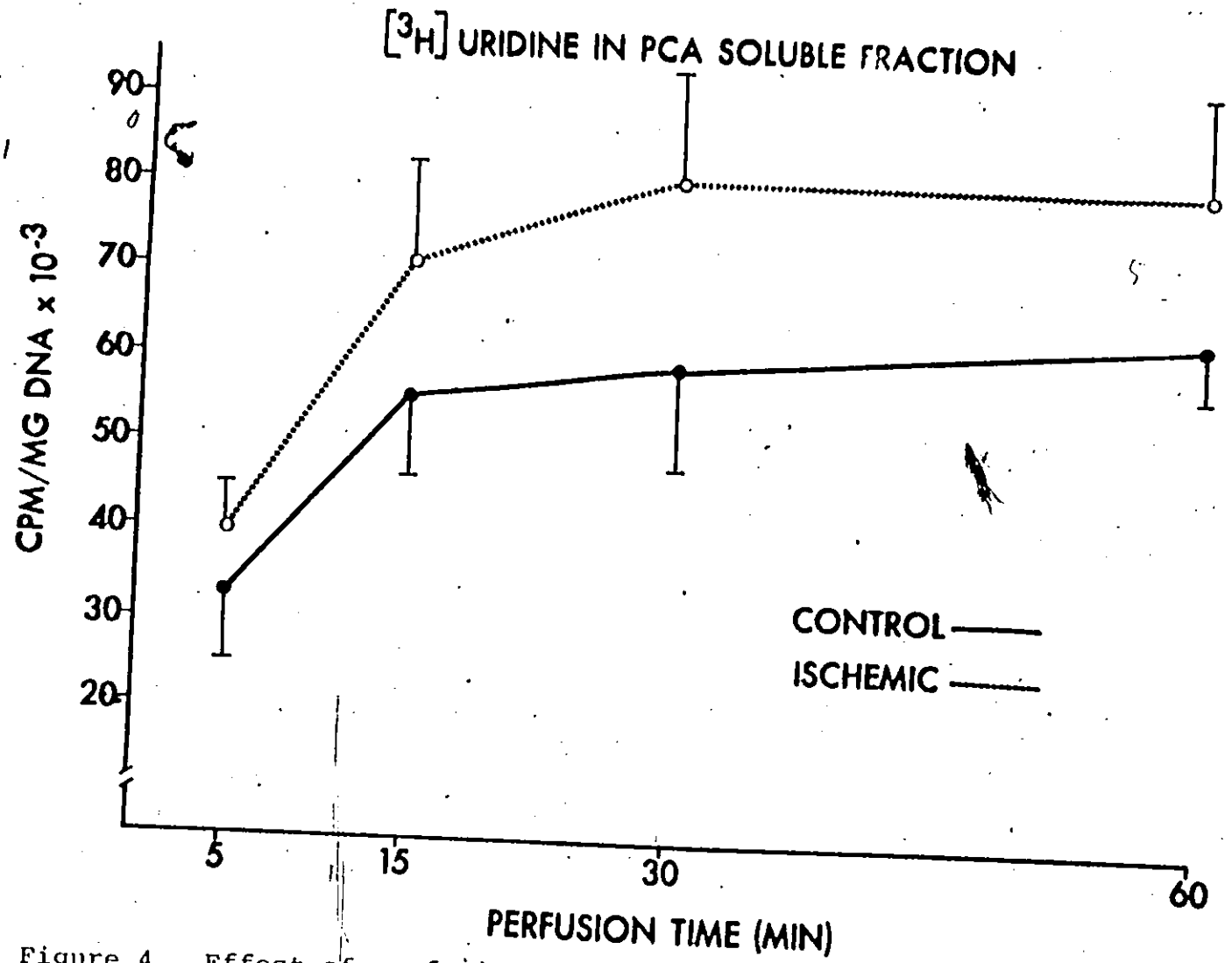


Figure 4. Effect of perfusion time on ($H^3$) uridine activity in the PCA soluble fraction of whole heart. Each value is mean ± S.E. of 4 animals.

reduction in uridine incorporation into RNA (Figure 5). The whole tissue uridine incorporation was depressed 24% following a similar 30 minute reperfusion (Figure 2). These data seem to suggest the occurrence of a severe disruption of the transcriptional process specifically in the cardiac muscle cells during the initial 30 minutes following ischemia.

($^3$H) Uridine in the PCA Soluble Fraction in Isolated Myocytes

Myocytes were isolated and the PCA soluble fraction determined as previously described. In contrasts to the total tissue PCA soluble fraction (Figure 4), the myocytes from the control hearts had 27,292 cpms/mg DNA in the acid soluble fraction while the ischemic myocyte had 12,084.4 cpms/mg DNA (Figure 6). This represents a 55% reduction ($P < .001$) in the availability of free uridine in the ischemic myocytes.

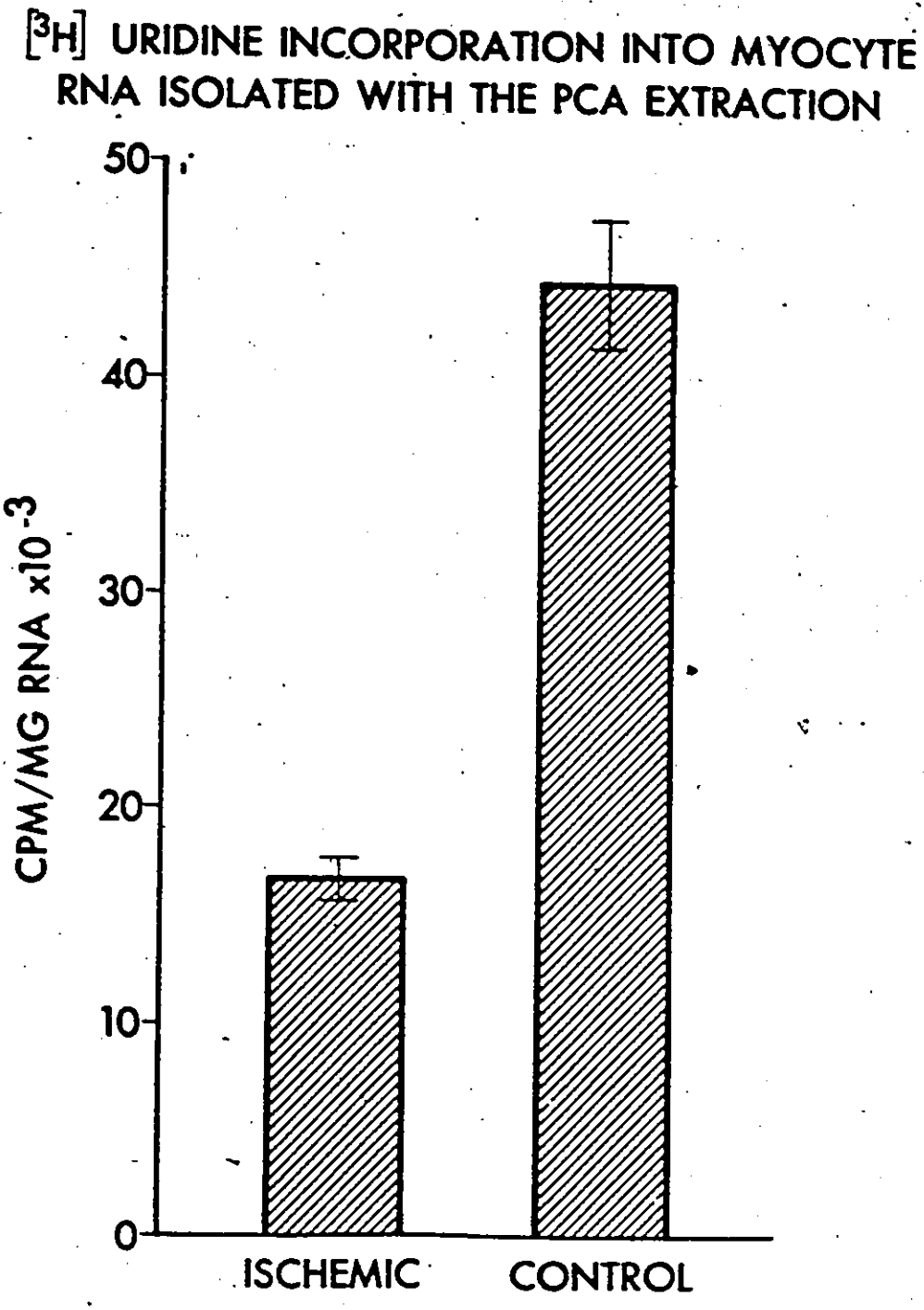


Figure 5. Hearts were perfused for 30 minuts. Two hearts were pooled for myocyte and RNA isolation. Each value is mean ± S.E. of 4 animals.

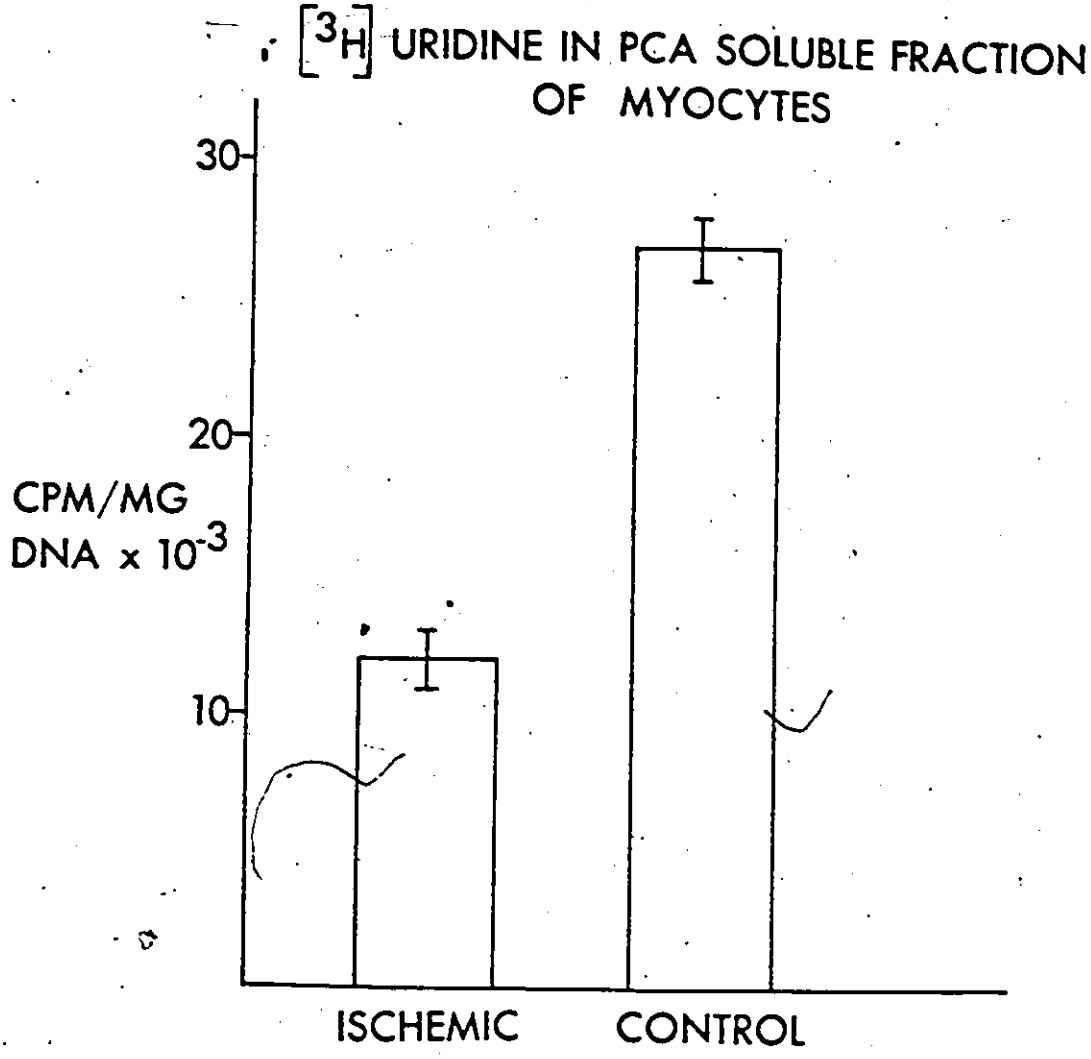


Figure 6. Hearts were perfused for 30 minutes. Two hearts were pooled for myocyte and PCA soluable fraction isolation. Each value is mean ± S.E. of 4 animals.

# CHAPTER IV

## DISCUSSION

Several studies have shown that chronic physiologic stress can result in an increase in myocardial nucleic acid and protein synthesis (2, 3, 4, 8, 10, 11, 16, 18, 25, 32, 33, 36, 41, 42, 45). To determine the independent contribution of work load and oxygen in these situations, the two variables have been independently altered. These studies have indicated that a decrease in oxygen supply can stimulate celluar transcription and translation (2, 11, 14, 18, 33). Therefore, it has become apparent that both factors can play an integral role in the initiation of RNA and protein synthesis. In the present study, 15 minutes of global ischemia, a condition of reduced oxygen availability because of a reduced perfusate flow to the heart was used to alter RNA synthesis. The hearts were made ischemic and reperfused with a buffer containing tritiated uridine. Fanburg et al. (10) have shown that during short term labelling studies this precursor is superior to orotic acid and uracil as it equilibrates with the intracelluar space at a faster rate and is converted to the immediate precursor at a higher rate.

Previous studies have shown that during the initial periods of hypoxia and anoxia (21) protein synthesis was reduced, possibly reflecting a disruption in energy (ATP) availability. Upon reoxygenation, protein synthesis quickly returns to control levels (40). In a study utilizing 30 minutes of hypoxic perfusion, Gibb (11) has shown an 80% increase in uridine incorporation during a 30 minute aerobic reperfusion period. Recently, Kleitke et al. (22) have shown a stimulation of mitochondrial RNA synthesis following an ischemic period. After no flow ischemic incubation, heart mitochondria incorporated both uridine and leucine at a rate of 120% and 35% above control. Collectively, these data support the notion that both ischemia and hypoxia may stimulate gene activation with subsequent RNA synthesis.

Various invivo and invitro techniques have been used to examine metabolic responses to myocardial ischemia and reperfusion. Surgically induced ligation of the circumflex branch of the left coronary artery is often used to produce regional ischmia affecting a limited amount of tissue (1, 16, 20, 25) while tissue incubation (22), autolysis (51), and the working heart preparation (50) have been used as invitro models. In the present study global myocardial ischemia was used

rather than regional ischemia in an attempt to magnify the subcellular response and provide a sufficient amount of tissue for the biochemical analysis. In addition, global myocardial ischemia is easily administered and provides a great deal of control.

Previous studies (1, 19, 20, 24, 43) have clearly shown that the duration of the ischemic interval is critical in determining the degree of reversible or irreversible damage. Irreversible damage is generally characterized by mitochondrial disruption, necrotic damage and severe enzymatic leakage due to membrane disruption (24). After a period of 20 to 40 minutes of ischemia, irreversible damage has been reported (20) but Schaper (43) has shown that the slight structural and metabolic changes evident after 15 minutes of global ischemia are quickly reversed upon reperfusion. In the present study, 15 minutes of ischemia was selected to reduce the possibility of tissue damage. The data in Table I clearly shows that the contraction rates and coronary flow quickly returned to normal following reperfusion and that all hearts were able to maintain 60 mmHg perfusion pressure. This suggests that hemodynamically, these hearts were not severely damaged and that the changes in RNA synthesis were probably not a result of alteration in ventricular performance during the recovery period.

In the initial stages of the present study, a high degree of variability was encountered. The phenol SDS isolation method (10) produced results exhibiting variable yields and uridine incorporation rates (Table 3). Fanburg (10) has stated that variable amounts of 4s RNA is probably extracted. Recently, a report by Lizardi et al. (28) criticized the phenol extraction procedure for disproportionate sampling of the smaller newly synthesized RNA species. From these observations, the initial data presented in this paper should be considered with caution as the phenol extraction has resisted validation.

Acid or base hydrolysis of nucleic acid has been a useful tool for the study of RNA and DNA in that they extract a higher percentage of total nucleic acid content. In comparing the relative activities of the RNA extracted with the phenol and perchloric acid procedures (Figure 2) there appeared little difference. Initially, the utilization of the phenol extraction identified a slight reduction in uridine incorporation following 60 minutes of reperfusion (Figure 1). But due to the obvious drawbacks later found associated with this procedure, the subsequent experiments were carried out using the PCA extraction. There was an apparent reduction in uridine incorporation at 30 ($P<.05$) and 60 ($P<.01$) minutes of ischemic reperfusion (Figure 3). These decreases

may reflect possibly changes in; a) uridine membrane transport, b) DNA depression, or c) nucleotide metabolism. The latter occurs during prolonged depletion of high energy phosphates (26) and is not believed to play a significant role in the present study as others have noted that ATP levels return to near normal following ischemic reperfusion (43). In contrast to the reduction in uridine incorporation, the PCA soluble fraction from the whole heart preparation appeared to be elevated in the ischemic hearts throughout the entire perfusion period. This large change in the PCA soluble fraction which was not statistically significant could be due to a change in the relative size of the extracellular space (37) or to an error in methodology as the extracellular space was not washed with non-radioactive buffer following the perfusion period. The reliability of the data in Figure 4 needs further investigation.

A major problem encountered in studies concerning myocardial genetic expression is the relative degree of tissue heterogeneity. Seventy percent of the heart is composed of non-muscle elements including vascular, endothelial and epithelial cells, fibroblasts and smooth muscle cells (7, 34). The cells of the

mammalian myocardium differ in metabolic activities, growth rates and response to environmental stimuli (7) which makes experimental interpretation difficult. The recent development of pure cell preparations allows clear identification of subcellular adaptations specific to cell types (7, 12, 15, 35).

Enzymatic digestion (12) of 30 minute control and ischemic reperfused hearts was used in the present study. The cells exhibited a high degree of homogenity and 80% were intact as measured by dye exclusion. Due to the size of the myocyte, initial attempts at homogenization with a glass teflon homogenizer proved unsuccessful but the use of the polytron yielded workable amounts of RNA.

Incorporation of ($^3$H) uridine into RNA was depressed 62% in the ischemic myocytes when compared to the control cells (Figure 5). This suggests that RNA synthesis was altered or possibly that there was a disruption of uridine membrane transport leading to a reduction in precursor available for RNA synthesis. The data in Figure 6 shows that free intracellular radioactivity was reduced 55% in the ischemic myocytes. This suggests that the transport mechanism for uridine was severely disrupted following 30 minutes of ischemic reperfusion. The depression in incorporation in the ischemic reperfused myocytes could have been magnified

by a reduction in template activiation and/or RNA polymerase activity but these factors need further investigation.

Studies involving long term coronary artery ligation result in compensatory hypertrophy. Gudbjarnasson (16) found an initial decrease in protein synthesis following coronary ligation followed by an increase in both protein and RNA synthesis. Lochner (27, 28) concluded that necrotic cell damage occurred prior to an increase in RNA and DNA synthesis in the ischemic heart. Gould has concluded that the initial increase in myocardial hypertrophy is associated with a normalization of ventricular wall tension (13). This information seems to suggest that the myocardial hypertrophy following pathologic ischemia (13, 27) is a secondary response to cell death.

A disruption in oxygen and substrate availability in the present study proved to be a depressor of RNA synthesis particularly in the ischemic myocyte. This observation can be partially explained by the observed decrease in intracellular uridine. From present and previous observations, there appears to be a conflict in the importance of oxygen availability following ischemia and hypoxia. To determine whether oxygen availability is principally involved in genetic

activation, further work is needed. This study also underlines the importance of the use of homogeneous cell populations during biochemical analysis. To gain a clear understanding of the effect of global ischemia, studies using pure non-muscle and muscle cell populations could clarify the descrepancies noted between whole heart and muscle cell preparations noted in the present study.

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# APPENDIX A

## Perfusion Buffer

### 1. Krebs, Henseleit Bicarbonate Buffer (24)

| Salt Stocks | %(gm/100ml) | Molar Conc. | ml Used (x3) |
|---|---|---|---|
| NaCl | .9 | .154 | 1000 (x3) |
| KCl | 1.15 | .154 | 40 (x3) |
| $CaCl_2$ | 1.22 | .110 | 36 (x3) |
| $KH_2PO_4$ | 2.11 | .154 | 10 (x3) |
| $MgSO_4 .7H_2O$ | 3.82 | .154 | 10 (x3) |
| $Na_2EDTA .2H_2O$ | 3.72 | .100pH7.4 | 6 (x3) |
| $NaHCO_3$ | 1.30 | .154 | 6 (x3) |
| Substrate (Glucose) | .391 | | |

### 2. Procedure for Buffer Preparation

Add 27gm NaCl ) - Dilute to 3 litres with
11.8gm Glucose ) double distilled water.

Add Appropriate volumes of stock salt sol'n ($KCl$, $CaCl_2$, $KH_2PO_4$, EDTA)

Gas with 95% $O_2$ & 5% $CO_2$ for 5 - 10 minutes.

Add Appropriate vol. of $NaHCO_3$ slowly while buffer is being oxygenated.

# APPENDIX B

## ISOLATION OF RNA (METHOD I)

Fanburg (10)

1. Buffers

Sol'n A - 20mM TRIS-HCl (pH 7.2)

Sol'n B - 88% Phenol
0.1% (W/V) 8-OH Quinoline

Sol'n C - 2·4M $CH_3$ COONa

Sol'n D - .01M TRIS-HCL (pH 7·2)

Sol'n E - .244M $CH_3$ COONa

Sol'n F - 4M $CH_3$ COOK

2. Procedure

Pulse label hearts

Trim atria, fat and connective tissue and weight

Mince and homogenize heart in 15ml of Sol'n A

Make homogenate 0.5% (W/V) with respect to SDS, Vortex

Let Stand 5 minutes

Add 15ml of Sol'n B and shake on ice for 30 min.

Spin at 10,000 x g for 30 min.

Remove upper aqueous phase containing RNA and add 10ml of Sol'n B to upper phase

Shake on ice for 30 min.

Spin at 10,000 x g 10 min.

Remove upper aqueous phase and to it add 1/10 volume Sol'n C and 2.5 volumes of 95% ethanol

Let stand 2 hours at -20° C.

Spin at 10,000 x g 10 min.

Aspirate and suspend pellet in 3ml of Sol'n D, add 30ug DN'ase I and wait 15 min. at room temp.

Add 7ml of ice cold distilled water and make 0.5% SDS (W/V) with respect to SDS

Add 5ml Sol'n B and shake on ice for 15 min.

Spin at 10,000 x g 10 min.

To upper aqueous phase add 1/10 volume of Sol'n C and 2.5 volumes of 95% ethanol

Let stand 2 hours at -20° C.

Spin at 10,000 x g 10 min.

To pellet add 2ml Sol'n E, 2ml of Sol'n F and 1ml 95% ethanol

Let stand 30 min. at -20° C.

Spin at 10,000 x g 10 min.

To pellet add 2ml Sol'n E, 2 ml of Sol'n F and 1ml 95% ethanol

Let stand 30 min. at -20° C.

Spin at 10,000 x g 10 min.

Transfer pellet to conicle tube and wash 3x with 95% ethanol

Dry pellet

Suspend pellet in 1ml Sol'n E.

## ISOLATION OF RNA (METHOD II)

Maggio (31)

1. Procedure

Pulse label heart

Trim atria, fat and connective tissue and weigh

Mince and homogenize heart in 4ml of ice cold distilled water

Remove 1ml and to it add 4ml of 0.5N PCA

Let stand on ice for 15 min. then spin at 600 x g for 5 min.

Wash pellet with 5ml of 0.5N PCA spin at 3,000 x g

Wash pellet 2x with absolute ethanol, spin at 3,000 x g

Wash pellet with ether, spin at 3,000 x g

Extract nucleic acids with 2ml of .05N PCA at 80° C. for 30 min.

Spin at 3,000 x g, remove and save supernatant

Re-extract pellet with 2ml 0.5N PCA at 80° C. for 30 min.

Spin at 3,000 x g

Combine supernatant from each extraction.

# APPENDIX C

# ISOLATION OF MYOCYTES

Glick (12)

1. Buffers

Sol'n A - 116mM NaCl
- 5mM KCl
- 4mM Glucose
- .4mM $NaH_2 PO_4 H_2O$
- pH adjusted to 7.4 with 200mM $Na_2 HPO_4$

Sol'n B - Sol'n A
- Add 1mg/ml collagenase and 2mg/ml Hyaluronidase

2. Procedure

Pulse label heart

Trim atria, fat and connective tissue and weigh

Cut ventricles into $2mm^2$ cubes

Wash 3x with Sol'n A

Place 1gm tissue in 25ml erlenmeyer flask, add 3.5ml Sol'n B

Shake flask at 100 strokes per minute in temp. controlled bath (37° C.) for 30 min.

Add 10ml Sol'n A, filter through fine nylon mesh into test tube on ice

Return undigested chucks to the flask and add 3.5ml of Sol'n B, incubate with shaking at 37° C. for 30 min. This process is repeated a total of 5x. The 1st digestion was discarded while the last four digestions were pooled to harvest myocytes.

After last digestion, spin at 37 x g for 5 min.

Wash pellet 3x with Sol'n A spinning at 37 x g for 5 min.

Add 3ml Sol'n A to pellet

Layer sample onto 10ml of ice cold 3% sucrose. Spin at 60 x g for 5 min.

Wash pellet 3x with Sol'n A, spinning at 37 x g for 5 min.

Store at -30° C.

# APPENDIX D

## RNA DETERMINATION

Schneider (44)

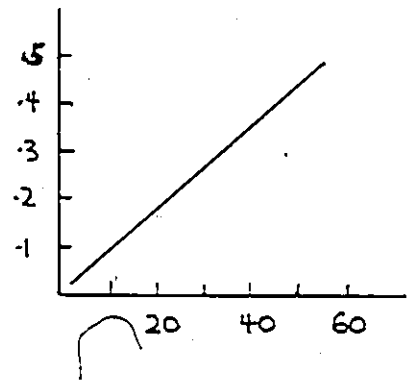
A) Chemicals
1) Orcinol (1gm/100ml HCl)
2) $FeCl_2$ (.5gm/100ml HCl)
3) Reagent HCl (12N)

B) Standard — Yeast RNA (1mg/ml in .244M Na Acetate)

C) Sol'n A — Prepare Fresh Daily
1) .5gm orcinol
2) .25gm $FeCL_2$
3) 50ml HCl

D) Assay
1) Make sample vol up to 1.5ml with double distilled water
2) Add 1.5ml Sol'n A
3) Place in boiling water for 30 min.
4) Cool for 10 min.
5) Read O.D. at 660nM.

E) Standard Curve



# APPENDIX E

## DNA DETERMINATION

Burton (5)

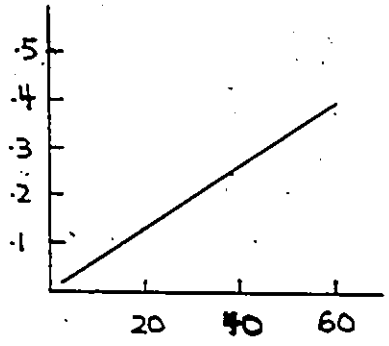
A) Chemicals
1) Diphenylamine
2) Reagent Grade $H_2SO_4$
3) Aqueous Acetaldehyde (16mg/ml)
4) 5mM NaOH
5) Glacial Acetic Acid

B) Standard
1) Calf thymus DNA = 2mg/ml in 5mM NaOH (Stored at -30° C)
2) Hydrolyze equal volume of DNA std. and .05N PCA for 15 min. at 70° C (Prepare fresh daily)

C) Diphenylamine Reagent
1) 1.5g Diphenylamine in 100ml of glacial acetic acid
2) Add 1.5ml $H_2SO_4$
3) At time of assy add 0.10ml of aqueous acetaldehyde/20ml of the diphenylamine, $H_2SO_4$ mixture and stir

D) Assay
1) Make sample vol. up to 1ml with 0.5N PCA
2) Add 2ml of Diphenylamine Reagent and Vortex
3) Incubate 20 hrs. at 30° C.
4) Read O.D. at 600nM

E) Standard Curve



# APPENDIX F

## EFFECT OF PERFUSION TIME ON ($^3$H) URIDINE INCORPORATION INTO RNA MEASURED BY METHOD I (CPM/MG RNA)

### Ischemic

| 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|
| 6,282·6 | 16,455·3 | 30,121·6 | 23,395·4 |
| 3,320·6 | 19,199·9 | 18,602·8 | 28,801·8 |
| 4,283·9 | 21,532·6 | 17,647·5 | 19,601·8 |
| 4,939·9 | 17,625·7 | 22,766·8 | 24,825·3 |
| | | 29,293·6 | 21,519·1 |
| | | 22,928·7 | 28,982·9 |
| | | 16,185·9 | 16,323·8 |
| | | 32,836·9 | |
| $\bar{x}$ = 4,706.8 ± 621.7 | $\bar{x}$ = 18,703.4 ± 1,098.2 | $\bar{x}$ = 23,798.4 ± 2,221.9 | $\bar{x}$ = 23,350.0 ± 1,762.1 |

### Control

| 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|
| 8,230·2 | 23,217 | 30,558.7 | 22,763.5 |
| 3,926·1 | 17,160·1 | 17,145·2 | 20,626 |
| 3,778·6 | 13,295·4 | 22,418·9 | 33,630·1 |
| 5,477·6 | 20,384·9 | 23,874·9 | 24,732·3 |
| | | 24,639·3 | 25,781·3 |
| | | 22,957·1 | 29,446·7 |
| | | 29,960·5 | 31,910·3 |
| | | 17,282·4 | 31,931·3 |
| $\bar{x}$ = 5,353.1 ± 1,033.1 | $\bar{x}$ = 18,514.4 ± 2,134.7 | $\bar{x}$ = 23,593.1 ± 1,756.4 | $\bar{x}$ = 27,602.7 ± 1,693.9 |

* Mean values are ± S.E.M.

## EFFECT OF PERFUSION TIME ON ($^3$H) URIDINE INCORPORATION INTO RNA MEASURED BY METHOD II

Ischemic

| 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|
| 8,094 | 13,325.4 | 17,029.9 | 29,474.1 |
| 7,589.4 | 12,566.5 | 13,926.9 | 34,213.1 |
| 6,531 | 9,649.9 | 18,652 | 32,431.7 |
| 7,132.7 | 13,339.2 | 17,850.9 | 32,877.4 |
| $\bar{x}$ = 7,336.8 ± 407.5 | $\bar{x}$ = 12,220.3 ± 875.6 | $\bar{x}$ = 16,864.9 ± 1,033.8 | $\bar{x}$ = 32,249.1 ± 999.4 |

Control

| 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|
| 6,757.4 | 16,679 | 19,958.2 | 43,745.3 |
| 7,380.9 | 11,543.2 | 24,143.7 | 39,316.5 |
| 6,021.9 | 13,238.2 | 19,861 | 44,632.6 |
| 5,220.8 | 14,282.4 | 25,841.9 | 46,160.6 |
| $\bar{x}$ = 6,345.3 ± 466.5 | $\bar{x}$ = 13,935.7 ± 1,074.6 | $\bar{x}$ = 22,451.2 ± 1,507.9 | $\bar{x}$ = 43,463.8 ± 1,469.6 |

* Means values are ± S.E.M.

DATA FROM PCA AND PHENOL EXTRACTION METHODS
(30 MIN. PERFUSED LABELLED HEARTS, CPM/MG RNA)

| PCA | PHENOL |
|---|---|
| 26,469.3 | 26,036 |
| 24,320.1 | 29,555.2 |
| 23,666.6 | 24,612.4 |
| 23,018.6 | 21,127.5 |
| 24,727.3 | 26,470.8 |
| 24,098.6 | 24,188 |
| 19,500 | 23,407.5 |
| 18,997.4 | |
| $\bar{x}$ = 23,099.7 ± 912.3 | $\bar{x}$ = 25,056.8 ± 1,003.6 |

* Means values are ± S.E.M.

URIDINE INCORPORATION INTO RNA ISOLATED FROM MYOCYTES*

| Control | Ischemic |
|---|---|
| 38,509.8 | 19,387.8 |
| 51,983.1 | 16,283.6 |
| 41,104.3 | 15,253.4 |
| 44,652.8 | 16,285.9 |
| $\bar{x}$ = 44,062.5 ± 2,925.1 | $\bar{x}$ = 16,802.6 ± 895.3 |

*Means values are ± S.E.M.

* All hearts were perfused for 30 min. with ($^{3}H$) uridine.

# APPENDIX G

## RESULTS OF STATISTICAL ANALYSIS

### ($^3$H) Uridine Incorporation into RNA (Method I)

| | 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|---|
| t | -.536 | .079 | .073 | .174 |
| D.F. | 4.983 | 4.540 | 15.112 | 14.831 |
| P | >.05 | >.05 | >.05 | >.05 |

### ($^3$H) Uridine Incorporation Into RNA (Method II)

| | 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|---|
| t | 1.731 | -1.238 | -3.056 | -6.312 |
| D.F. | 5.424 | 5.703 | 5.333 | 5.341 |
| P. | >.05 | >.05 | <.05 | <.01 |

### Free ($^3$H) Uridine in PCA Soluble Fraction of Total Tissue

| | 5 Min. | 15 Min. | 30 Min. | 60 Min. |
|---|---|---|---|---|
| t | .756 | 1.000 | 1.750 | 1.753 |
| D.F. | 6.301 | 6.937 | 4.325 | 5.432 |
| P. | >.05 | >.05 | >.05 | >.05 |

### ($^3$H) Uridine Incorporattion into RNA of Isolated Myocytes

| | 30 Min. |
|---|---|
| t | 8.911 |
| D.F. | 3.930 |
| P | <.001 |

Free ($^3$H) Uridine in PCA Soluble Fraction of Isolated Myocytes

| | 30 Min. |
|---|---|
| t | -10.810 |
| D.F. | 5.937 |
| P | <.001 |

Comparison of ($^3$H) Uridine Incorporation Using Method I and Method II

| | |
|---|---|
| F | 2.09 |
| D.F.$_1$ | 1 |
| D.F.$_2$ | 3 |
| P | >.05 |

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